The administration of soluble myelin proteins is an effective way of down-regulating the inflammation in the central nervous system (CNS) in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis. To shed more light on the mechanism of this antigen-specific therapy, we determined the effect of the intraperitoneal (i.p.) injection of soluble myelin basic protein (MBP) on T cell apoptosis in the CNS and peripheral lymphoid organs of Lewis rats with EAE induced by inoculation with MBP and complete Freund’s adjuvant. In particular we assessed the level of apoptosis of Vβ8.2+ T cells, which constitute the predominant encephalitogenic MBP-reactive T cell population in the Lewis rat. The daily i.p. injection of MBP for 3 days from the onset of neurological signs inhibited the further development of neurological signs of EAE. Using two-colour flow cytometry we found that a single i.p. injection of MBP increased the level of apoptosis of the Vβ8.2+ T cell population in the CNS to 26.2% compared to 7.4% in saline-treated rats and 7.6% in ovalbumin-treated rats. In contrast, treatment with MBP did not increase the level of apoptosis of the Vβ8.2+ population in the popliteal lymph node draining the inoculation site (1.4%) or in the spleen (1.6%) above that occurring in saline-treated rats (1.6% and 1.1%, respectively). Limiting dilution analysis revealed that the frequency of T cells reactive to the major encephalitogenic epitope, MBP72–89, was decreased in the CNS but not in the popliteal lymph node by this treatment. Three-colour flow cytometry in MBP-treated rats demonstrated that CNS Vβ8.2+T cells expressing Fas (CD95) and Fas ligand were highly vulnerable to apoptosis compared to Vβ8.2+T cells not expressing these proteins. We conclude that the i.p. injection of MBP increases the spontaneously occurring Fas-mediated activation-induced apoptosis of autoreactive T cells in the CNS in EAE and that this contributes to the therapeutic effect of the injection.

**Keywords:** Apoptosis ; T cell ; Experimental autoimmune encephalomyelitis ; Fas (CD95) ; Treatment
1. INTRODUCTION

Experimental autoimmune encephalomyelitis (EAE) is a T cell-mediated demyelinating disease of the central nervous system (CNS). It can be induced by inoculation with myelin antigens in CFA or by the passive transfer of T cells specific for myelin antigens or for the appropriate encephalitogenic peptides. The characteristic clinical picture in the Lewis rat with acute EAE induced by inoculation with myelin basic protein (MBP) and CFA (MBP-EAE) is rapid spontaneous recovery and resistance to the re-induction of EAE by active immunization [1]. Immunization of Lewis rats with MBP induces encephalitogenic T cells that predominantly use Vβ8.2 in their TCR [2, 3]. The pathogenic role of Vβ8.2+ cells in MBP-EAE is indicated by the inhibition of disease by treatment with a mAb against the Vβ8.2 segment of the TCR [4]. Furthermore, Vβ8.2+ T cells are the predominant T cell in the CNS at the onset of MBP-EAE [5–7].

In Lewis rats with EAE, T cells, especially Vβ8.2+ MBP-reactive T cells, are eliminated from the CNS by apoptosis, and this contributes to the spontaneous down-regulation of the disease [7–12]. This T cell apoptosis is largely dependent on the environment in the target organ of this autoimmune disease, as it occurs in the CNS but not in the peripheral lymphoid organs [9, 10]. We have hypothesized that it is due to activation-induced cell death of encephalitogenic T cells interacting with CNS non-professional APC such as astrocytes and microglia which fail to provide sufficient co-stimulation to up-regulate the T cell expression of anti-apoptotic proteins such as Bcl-2, which inhibits Fas (CD95)-mediated T cell apoptosis [7, 9, 13]. This hypothesis is supported by the following findings: (1) non-autoreactive T cells which accumulate together with autoreactive T cells but are not reactivated in the CNS during EAE, are not deleted but recirculate to the peripheral lymphoid organs [11]; (2) corticosteroids, which antagonize activation-induced apoptosis in vitro, inhibit the selective apoptosis of Vβ8.2+ T cells in the CNS [14]; (3) Vβ8.2+ T cells expressing Fas and Fas ligand (FasL), which can mediate activation-induced T cell apoptosis, are highly vulnerable to apoptosis in the CNS in EAE, whereas cells expressing Bcl-2, which is increased by co-stimulation, are relatively protected from apoptosis [13].

Being the best available model of the human demyelinating disease multiple sclerosis, EAE has been studied extensively to explore a variety of therapeutic modalities [15]. Antigen-specific down-regulation of EAE by the systemic administration of soluble myelin proteins or peptides after the onset of clinical disease has been studied for over 30 years [16–22]. The underlying mechanisms of such therapy have been attributed to clonal anergy [18, 22], immune deviation (a switch from Th1 to Th2 responses) [21], or peripheral deletion of autoreactive T cells [19, 20]. However, little is known about the fate of the autoreactive T cells in the target organ after these antigen-specific therapies. In the present study, we investigated the effect of i.p. injection of MBP on acute MBP-EAE in the Lewis rat and found that this treatment increases the level of apoptosis of Vβ8.2+ T cells in the CNS but not in the peripheral lymphoid organs. These findings suggest that parenterally administered MBP augments the spontaneously occurring activation-induced T cell apoptosis in the target organ of this autoimmune disease.

2. RESULTS

2.1 Clinical effect of i.p. MBP treatment on acute EAE

Rats with MBP-EAE were given i.p. injections of saline, 50 µg OVA, 100 µg OVA, 50 µg MBP or 100 µg MBP daily for 3 consecutive days commencing on day 11, at or just after the
onset of neurological signs. As shown in Fig. 1, MBP treatment reduced the total clinical score on day 14 and day 15 significantly and in a dose-dependent manner, confirming the inhibitory effect of i.p. MBP on EAE. Some of the rats developed minor relapses, but the severity of these was unrelated to the treatment.

Figure 1. Mean total clinical scores of rats with EAE treated with daily i.p. injections (arrows) of saline, 50 µg OVA, 100 µg OVA, 50 µg MBP or 100 µg MBP for 3 days commencing 11 days after inoculation with MBP and CFA (day 11). Twenty-five rats were divided into five groups of five with the same mean total clinical score on day 11 and checked by an examiner who was unaware of the treatment that each rat received. The mean total clinical scores of the five groups were compared on each day simultaneously using ANOVA, followed by Student’s t-test to compare the pairs of groups. The ANOVA p values for days 14 and 15 were 0.036 and 0.068, respectively. Student’s t-tests for day 14: MBP 100 µg vs. saline, p = 0.005; MBP 100 µg vs. OVA 100 µg, p = 0.003; MBP 100 µg vs. MBP 50 µg, p = 0.015. Student’s t-tests for day 15: MBP 100 µg vs. saline, p = 0.032; MBP 100 µg vs. OVA 100 µg, p = 0.020; MBP 50 µg vs. OVA 50 µg, p = 0.049. Some of the rats developed minor relapses, but the severity of these was unrelated to the treatment; the ANOVA p values on days 25, 26, 27 and 28 were 0.814, 0.534, 0.395 and 0.751, respectively.

2.2 Effect of i.p. MBP treatment on the level of T cell apoptosis in the CNS and peripheral lymphoid organs

To study the effect of i.p. MBP treatment on the levels of apoptosis of CD5+, TCR αβ + or Vβ8.2+ populations in the CNS, the draining lymph node and the spleen, we performed two-colour flow cytometric analysis (surface antigens and DNA staining) 12 h after a single i.p. injection of saline, OVA (100 µg or 500 µg) or MBP (100 µg or 500 µg) given on day 11 (Fig. 2). In saline-treated rats with EAE, the percentages of TCR αβ + cells and Vβ8.2+ cells in the spinal cord that were apoptotic were 7.9% and 7.4% respectively, similar to the results of our previous study [12]. In rats with EAE treated with 500 µg OVA, the percentages of TCR αβ + cells and Vβ8.2+ cells in the spinal cord that were apoptotic were 4.5% and 7.6%, respectively. In rats with EAE treated with 500 µg MBP, the percentages were significantly increased to 18.5% and 26.2%, respectively. The percentage of the total inflammatory cell population that
was apoptotic after treatment with 500 µg MBP was also significantly increased. Furthermore, the level of apoptosis of Vβ8.2+ T cells was significantly higher than the level of apoptosis of the total inflammatory cell population, indicating that autoreactive T cells were preferentially targeted in the spinal cord by treatment with 500 µg MBP. The 100-µg dose of MBP also tended to increase apoptosis in the spinal cord, but the effect was variable from experiment to experiment, and the means were not significantly different from those of controls (Fig. 2a).

In saline-treated rats the percentages of TCR αβ+ cells and Vβ8.2+ cells that were apoptotic in the draining lymph node were 1.3% and 1.6%, respectively, and in the spleen 1.2% and 1.1%, confirming earlier reports that T cell apoptosis in EAE is essentially restricted to the nervous system [9, 10]. MBP treatment did not increase the levels of T cell apoptosis in these lymphoid organs above those of saline-treated rats (Fig. 2b, c).

Fig. 3 shows a representative dot-plot analysis of the Vβ8.2+ cell population obtained from the spinal cords (a, d), the draining lymph nodes (b, e) or the spleens (c, f) of rats 12 h after the i.p. injection of 500 µg OVA (a, b, c) or 500 µg MBP (d, e, f). The apoptotic population was defined as having a lower FL2 signal [propidium iodide (PI)] than the well-defined G0/G1 population [7]. As expected, the cells in the thus defined apoptotic population were smaller [lower forward scatter (FSC)] than those in the G0/G1, S and G2/M populations (Fig. 3). The numbers of total inflammatory cells and Vβ8.2+ T cells in the spinal cord in rats treated with 500 µg MBP tended to be lower than in saline-treated rats but the differences were not statistically significant (Table 1).

![Figure 2](image-url)  
Figure 2. Percentages of CD5+, TCR αβ+ and Vβ8.2+ cells that were apoptotic in the spinal cord (a), draining lymph node (b) and spleen (c) of rats with EAE 12 h after the i.p. injection of saline, OVA or MBP (given on day 11). Treatment with 500 µg MBP significantly increased the level of apoptosis of the total inflammatory cell population (total), αβ T cells and Vβ8.2+ T cells in the spinal cord, but not in the draining lymph node or spleen. MBP 500 µg vs. saline: total, p = 0.0005; CD5+, not significant (p G 0.05); TCR αβ+, p = 0.0042; Vβ8.2+, p = 0.0006. Furthermore, the level of apoptosis of Vβ8.2+ T cells was significantly higher than the level of apoptosis of the total inflammatory cell population in the spinal cord after treatment with 500 µg MBP (p = 0.016). The number of groups of rats studied for each treatment was as follows: saline, 3–4; 100 µg OVA, 1–2; 500 µg OVA, 1–2; 100µg MBP, 4–5; 500 µg MBP, 3–4. Not all organs were studied in each group.
2.3 Limiting dilution analysis of the encephalitogenic cells

To determine whether the deletion of Vβ8.2+ T cells in the CNS is reflected in a decrease in the frequency of T cells proliferating in response to the encephalitogenic MBP_{72-89} peptide, we performed limiting dilution analysis on the spinal cord inflammatory infiltrate, the draining lymph node and the spleen of rats 12 h after treatment with a single i.p. injection of either saline or 500 µg MBP given on day 11. As shown in Table 2, the MBP treatment did not alter the frequencies of T cells reactive to MBP or to the major encephalitogenic epitope, MBP_{72-89}, in the draining lymph node. There was a decrease in the frequency of MBP-reactive cells in the spleen after MBP treatment but the significance of this is uncertain because the frequency of Con A-reactive cells in the spleen was also reduced. The frequency of MBP_{72-89}-reactive cells in the spleen was below the limit of sensitivity of the assay in saline-treated and MBP-treated rats. In the spinal cord, the frequencies of MBP-reactive and MBP_{72-89}-reactive cells were considerably decreased by the MBP treatment.
Table 1. Cell recovery from the spinal cords of rats with EAE after i.p. MBP treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of groups studied</th>
<th>Mean total clinical score (day 12)</th>
<th>Mean cell yield from the spinal cord</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total[^a]</td>
</tr>
<tr>
<td>Saline</td>
<td>4</td>
<td>4.3 ± 2.3</td>
<td>3.79 ± 2.36[^b]</td>
</tr>
<tr>
<td>MBP 100 µg</td>
<td>4</td>
<td>4.3 ± 2.0</td>
<td>4.20 ± 3.12</td>
</tr>
<tr>
<td>MBP 500 µg</td>
<td>5</td>
<td>3.5 ± 2.0</td>
<td>1.98 ± 1.02[^c]</td>
</tr>
</tbody>
</table>

a) Rats with EAE were treated on day 11 and killed 12 h later.
b) × 10^6/gram of spinal cord before nylon wool column enrichment.
c) × 10^5/gram of spinal cord after nylon wool column enrichment.
d) p = 0.08 (Student’s t-test).
e) p = 0.08 (Student’s t-test).

Table 2. Frequency of Con A-, MBP- and MBP[^2,40]-reactive cells in rats with EAE

<table>
<thead>
<tr>
<th>Popliteal lymph node</th>
<th>Spinal cord</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline[^a]</td>
<td>MBP 500 µg[^a]</td>
</tr>
<tr>
<td>Con A</td>
<td>1/31[^b]</td>
</tr>
<tr>
<td>MBP</td>
<td>1/28 000</td>
</tr>
<tr>
<td></td>
<td>(1/40 000–1/19 000)</td>
</tr>
<tr>
<td>MBP[^2,40]</td>
<td>1/45 000</td>
</tr>
<tr>
<td></td>
<td>(1/67 000–1/20 000)</td>
</tr>
</tbody>
</table>

a) Rats with EAE were treated with saline or 500 µg MBP on day 11 and killed 12 h later. There were seven rats in each treatment group.
b) Frequency.
c) 95% confidence limits.

2.4 Expression of apoptosis-regulating proteins by Vβ8.2+ cells

We have previously presented evidence that the apoptotic elimination of the disease-relevant Vβ8.2+ cells during spontaneous recovery from MBP-EAE is mediated by the Fas pathway [13]. To study the role of the Fas pathway in the increased Vβ8.2+ T cell apoptosis in the CNS caused by i.p. MBP treatment, we used three-colour flow cytometric analysis to stain simultaneously Vβ8.2, Fas/FasL and DNA as previously described [13]. The spinal cord inflammatory cells from rats with EAE were analysed 12 h after the i.p. injection of 500 µg MBP given on day 11. As shown in Table 3, the percentage of all cells that were Fas+ was not increased, whereas the percentage of all cells that were FasL+ was significantly increased by the treatment. The proportions of Vβ8.2+ cells that were Fas+ or FasL+ were not significantly increased. However, the proportion of Vβ8.2+Fas+ cells that were apoptotic and the proportion of Vβ8.2+FasL+ cells that were apoptotic were increased by more than threefold to 46.5% and 58.3%, respectively, by the treatment (Table 4).
In sharp contrast, the proportion of $\text{V}\beta 8.2^+\text{Fas}^-$ cells that were apoptotic and the proportion of $\text{V}\beta 8.2^+\text{Fas}^+$ cells that were apoptotic were not increased by i.p. MBP treatment. After MBP treatment the proportion of $\text{V}\beta 8.2^+\text{Fas}^+$ cells that were apoptotic was 10.9 times the proportion of $\text{V}\beta 8.2^+\text{Fas}^-$ cells that were apoptotic, and the proportion of $\text{V}\beta 8.2^+\text{FasL}^+$ cells that were apoptotic was 12.2 times the proportion of $\text{V}\beta 8.2^+\text{FasL}^-$ cells that were apoptotic (Table 4). The increased vulnerability of $\text{V}\beta 8.2^+\text{Fas}^+$ cells and $\text{V}\beta 8.2^+\text{FasL}^+$ cells to apoptosis probably accounts for our failure to detect significant increases in the proportion of $\text{V}\beta 8.2^+$ T cells expressing Fas or in the proportion of $\text{V}\beta 8.2^+$ T cells expressing FasL after MBP treatment (Table 3). These findings indicate that i.p. MBP treatment augments $\text{V}\beta 8.2^+$ T cell apoptosis through the Fas pathway.

3. DISCUSSION

In the present study we have shown by two-color flow cytometry that i.p. treatment with soluble MBP increases the levels of apoptosis of $\alpha\beta$ T cells and $\text{V}\beta 8.2^+$ T cells in the CNS but not in the peripheral lymphoid organs of rats with MBP-EAE. $\text{V}\beta 8.2^+$ T cells constitute the predominant encephalitogenic MBP-reactive T cell population in the Lewis rat [4]. Using limiting dilution analysis we have also demonstrated that the frequency of T cells reactive to the major encephalitogenic epitope, MBP$_{72-89}$, is decreased in the CNS but not in the lymph node by this treatment. These results indicate that soluble MBP therapy induces the apoptotic elimination of autoreactive T cells preferentially in the target organ rather than in the peripheral lymphoid organs. It is likely that the treatment with MBP also increases the apoptosis in the CNS of MBP-reactive T cells not expressing $\text{V}\beta 8.2$. Three-color flow cytometry revealed that CNS $\text{V}\beta 8.2^+$ T cells expressing Fas and FasL were highly vulnerable to apoptosis compared to $\text{V}\beta 8.2^+$ T cells not expressing these proteins and that $\text{V}\beta 8.2^+$ cells expressing these proteins were at least three times more vulnerable than in untreated rats with EAE. This indicates that soluble MBP therapy augments the previously described spontaneously occurring Fas-mediated activation-induced apoptosis of autoreactive T cells in the CNS in EAE [13]. The rapid (within 12 h) induction of increased apoptosis after MBP treatment is consistent with mediation through Fas/FasL interactions rather than through the action of TNF, which induces apoptosis in mature T cells more slowly than Fas ligation [23]. The demonstrated therapeutic effect of three i.p. injections of MBP on the clinical course of EAE can be explained by the increased apoptosis of encephalitogenic T cells in the CNS.

Table 3. Expression of Fas/FasL on the spinal cord inflammatory cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>None$^{a)}$</th>
<th>MBP 500 $\mu$g$^{b)}$</th>
<th>$p$ (t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of all cells that are Fas$^+$</td>
<td>5.87 ± 1.50</td>
<td>6.10 ± 1.03</td>
<td>0.44</td>
</tr>
<tr>
<td>% of $\text{V}\beta 8.2^+$ cells that are Fas$^+$</td>
<td>8.03 ± 4.18</td>
<td>9.61 ± 1.09</td>
<td>0.27</td>
</tr>
<tr>
<td>% of all cells that are FasL$^+$</td>
<td>3.42 ± 0.83</td>
<td>5.78 ± 1.11</td>
<td>0.04</td>
</tr>
<tr>
<td>% of $\text{V}\beta 8.2^+$ cells that are FasL$^+$</td>
<td>6.14 ± 5.68</td>
<td>7.09 ± 0.86</td>
<td>0.43</td>
</tr>
</tbody>
</table>

a) Two groups of six rats were studied.
b) Three groups of six to seven rats were studied.
a) One group of six rats with EAE was studied.
b) Two groups of six to seven rats were studied. Rats with EAE were given 500 µg MBP on day 11 and killed 12 h later.
c) For each experiment the percentage of apoptotic cells in the population expressing Vβ8.2 and the apoptosis regulating protein was divided by the percentage of apoptotic cells in the population which were Vβ8.2+ but which did not express the protein. The mean and population standard deviation of these values were then calculated.

Our results shed light on the mechanism of soluble antigen therapy in autoimmune disease and may explain the discrepancies among the results of previous studies. Critchfield et al. [19] found that very high doses of MBP given i.v. and repeatedly to mice with EAE resulted in the deletion of autoreactive T cells from the peripheral lymphoid organs but they did not determine whether the treatment affected T cell deletion in the CNS. In contrast, Marusic and Tonegawa [22] found that a single injection of MBP peptide exerted a beneficial effect on EAE without deleting T cells in the spleen. They concluded that the therapeutic effect of soluble antigen administration was due to peripheral T cell anergy but they did not assess T cell deletion in the CNS. In the present study there was no evidence of peripheral T cell anergy after i.p. treatment with soluble MBP, as there was no significant decrease in the frequency of lymph node T cells specifically proliferating in response to MBP72–89. Furthermore, we have previously shown that deletion, not anergy, is responsible for the T cell unresponsiveness to MBP72–89 during spontaneous recovery from EAE [11].

We propose that soluble autoantigen therapy inhibits autoimmune disease by inducing apoptosis of autoreactive T cells preferentially in the target organ rather than in the peripheral lymphoid organs. Increasing the dose of administered antigen may induce apoptosis in the peripheral lymphoid organs but this may not be necessary for the therapeutic effect. This proposal is consistent with the results of Weishaupt et al. [24] who found that two i.v. injections of P2 protein increased the level of total T cell apoptosis in the peripheral nervous system and spleen of Lewis rats with experimental autoimmune neuritis. Why does soluble antigen therapy induce apoptosis of autoreactive T cells preferentially in the target organ rather than in the peripheral lymphoid organs, given that the systemic administration of antigen should result in the distribution of the antigen throughout lymphoid and non-lymphoid organs, including the CNS?

Clearly the environment in the CNS is more conducive to activation-induced T cell apoptosis than the environment of the spleen or the popliteal lymph node draining the site of inoculation with MBP and CFA. One possible explanation is that professional APC in the peripheral lymphoid organs provide high levels of co-stimulation with resultant high T cell levels of anti-apoptotic proteins such as Bcl-2, which inhibit Fas-mediated activation-induced T cell apoptosis, whereas the predominantly nonprofessional APC in the CNS are unable to provide sufficient co-stimulation to inhibit this apoptosis [7, 9, 13, 25, 26]. Autoreactive T cells in the

\[\begin{array}{|c|c|c|c|}
\hline
\text{Apoptosis-regulating protein} & \text{Treatment} & \text{\% of Vβ8.2+protein+ cells that were apoptotic} & \text{\% of Vβ8.2+protein- cells that were apoptotic} & \text{Mean ratio}\text{a} \\
\hline
\text{Fas} & \text{None}\text{a} & 14.3 & 4.1 & 3.5 \\
& \text{MBP 500 µg}\text{b} & 46.5 \pm 19.2 & 4.8 \pm 1.6 & 10.9 \pm 7.7 \\
\text{FasL} & \text{None} & 12.9 & 4.8 & 2.7 \\
& \text{MBP 500 µg} & 58.3 \pm 20.0 & 5.1 \pm 1.1 & 12.2 \pm 6.7 \\
\hline
\end{array}\]

a) One group of six rats with EAE was studied.
b) Two groups of six to seven rats were studied. Rats with EAE were given 500 µg MBP on day 11 and killed 12 h later.
c) For each experiment the percentage of apoptotic cells in the population expressing Vβ8.2 and the apoptosis regulating protein was divided by the percentage of apoptotic cells in the population which were Vβ8.2+ but which did not express the protein. The mean and population standard deviation of these values were then calculated.
CNS may also have higher levels of Fas and FasL expression than those in the peripheral lymphoid organs because they are more likely to have been repeatedly activated by antigen. In conclusion, we have shown that the systemic administration of soluble MBP increases the spontaneously occurring Fas-mediated activation-induced apoptosis of autoreactive T cells in the CNS in EAE. Knowledge of the different fates of autoreactive T cells in the target organ and peripheral lymphoid organs is likely to be important in understanding the pathogenesis of autoimmune diseases and in developing more effective treatment.

4 MATERIALS AND METHODS

4.1 Animals

Female Lewis rats (JC strain), 7–9 weeks old, were obtained from the Central Animal Breeding House of the University of Queensland. They were fed rat and mouse cubes and water ad libitum.

4.2 Preparation of inoculum and induction of EAE

MBP was prepared from frozen guinea pig brains by the method of Deibler et al. [27]. MBP in 0.9% saline (1 mg/ml) was emulsified in an equal volume of IFA (Sigma Immuno Chemicals) containing 4 mg/ml Mycobacterium butyricum (Difco). Under anesthesia with ketamine, xylazine and atropine, rats were inoculated with 0.1 ml of the emulsion in a footpad of the left hindfoot. The total dose of MBP was 50 µg/rat.

4.3 Assessment of clinical effect of i.p. MBP injections on EAE

Tail, hindlimb and forelimb weakness were separately graded on a scale of 0 (no weakness) to 4 (total paralysis) as previously described [28]. The total clinical score was obtained by adding these three scores (maximum = 12). Twenty-five rats were inoculated with MBP and CFA, and 11 days after inoculation (day 11) they were divided into five groups of five with the same mean total clinical score, and given i.p. injections of saline, 50 µg or 100 µg of OVA, or 50 µg or 100 µg of MBP for 3 consecutive days commencing on day 11. The clinical status was checked until day 40. The examiner was blinded to the type of treatment the individual rats received.

4.4 Preparation of T cells from the spinal cord and the lymphoid organs

To examine the effect of soluble MBP therapy on the level of apoptosis in the spinal cord, the draining lymph node (the left popliteal lymph node) and the spleen, rats were injected i.p. with saline, OVA or MBP once on day 11 and killed 12 h later on day 12. In most experiments, spinal cord inflammatory cells were obtained from groups of six animals, and spleen and lymph node cells were obtained from the two most severely affected animals in the same group. Spinal cord inflammatory cells were extracted as previously described [7]. Briefly, rats were perfused with ice-cold saline, the entire spinal cord was removed by insufflation and weighed, and a single-cell suspension in ice-cold RPMI 1640 containing 1% FCS was prepared by passage of the spinal cord through a 200-mesh stainless steel sieve. The cell suspension was mixed with isotonic Percoll (Percoll :HBSS = 9 : 1) in a 3 : 2 ratio in a 50-ml centrifuge tube and spun for 25 min at 640 × g at 4 °C. The cell pellet was resuspended in the last 9 ml of supernatant, transferred to a conical tube, underlaid with 1.5 ml Ficoll and spun for 20 min at 600 × g at 4 °C. Cells were collected from the interface and washed. Single-cell suspensions were prepared from the left popliteal lymph node and spleen by teasing and passage through a fine nylon mesh. Erythrocytes
were removed from the spleen cell suspension using Ficoll. Cells from the spinal cord, lymph node and spleen were enriched for T cells using a nylon wool column.

4.5 Antibodies and staining for flow cytometric analysis

Mouse mAb specific for CD5 (expressed by T lymphocytes) (OX19) and the αβ TCR (R73) were obtained from Dr. J. Sedgwick. Mouse mAb to Vβ8.2 (R78) [29] was a kind gift from Dr. T. Hünig. Rabbit polyclonal antibodies specific for rat Fas (M-20) and FasL (W-20) were purchased from Santa Cruz Biotechnology, Inc. Western blot analysis of the CNS inflammatory cell protein extract confirmed the specificity of antibody binding (not shown). Mouse polyclonal IgGl O (Dako) and rabbit IgG (Rockland) were used as control antibodies. The secondary antibodies were FITC-conjugated sheep F(ab’)2 anti-mouse IgG (Sigma), FITC-conjugated goat F(ab’)2 anti-rabbit IgG (Rockland) and PE-conjugated goat F(ab’)2 anti-mouse Ig (Dako). For two-colour flow cytometric analysis, cells were first washed with a 1 : 1 solution of FCS in PBS containing 0.1% sodium azide and then incubated with the primary antibodies for 30 min at 4 °C. Cells were washed, incubated with the secondary antibody for 30 min at 4 °C and, washed twice with PBS. Samples were resuspended in 1 ml ice-cold 50% ethanol and fixed overnight at 4 °C in the dark. The ethanol was removed by washing the cells with PBS, and the cells were resuspended in an appropriate volume (100–300 µl) of PI staining solution which was freshly prepared by diluting stock solution (5 mg/ml RNase/250 µg/ml PI in 0.01 M PBS containing 0.1 mM EDTA, pH 7.4) 1 : 4 with PBS containing 0.1% sodium azide. In some experiments, three-colour flow cytometric analysis was performed to examine simultaneously the DNA content and the expression of Vβ8.2 and Fas/FasL, as described previously [13]. Briefly, cells were first stained for expression of Vβ8.2 and Fas/FasL as for two-colour analysis. After washing to remove secondary antibodies, cells were fixed with 1 ml ice-cold 0.25% paraformaldehyde in PBS (pH 7.2) overnight at 4 °C. They were then washed in PBS and permeabilized by being gently resuspended in 1 ml 0.2% Tween-20 in PBS and incubated at 37 °C for 15 min. Samples were washed and resuspended in the PI staining solution. Samples were analysed using a FacsCalibur and CELLQuest software (Becton Dickinson). To analyse the level of apoptosis, histogram plots of PI fluorescence were obtained. Apoptotic events were defined as those having lower fluorescence than the sharply defined G0/G1 peak [7]. Electronic compensation ensured unchanged FITC and PE distribution following PI labeling of DNA. To avoid detecting nuclear debris, events with a low level of PI fluorescence were excluded from analysis. For each sample, 40 000 events were scored.

4.6 Limiting dilution analysis of the encephalitogenic cells

We used limiting dilutions analysis [11] to determine the frequencies of T cells reactive to MBP, MBP72–89 (sequence: PQKSQRSQDENPVVHF), the major encephalitogenic region of guinea pig MBP in the Lewis rat, or Con A in the spinal cord, the draining popliteal lymph node and the spleen of rats with acute EAE treated with i.p. MBP or saline. Cells were prepared as described under Sect. 4.4. The culture medium used for this assay was RPMI 1640 supplemented with 216 mg/l L-glutamine (Gibco), 100 IU penicillin, 100 µg/ml streptomycin, 0.1 mM sodium pyruvate, nonessential amino acids, 36 mg/l L-asparagine, 5 × 10⁻⁵ M 2-ME, 5 µg/ml fungizone and 5% heat-inactivated FCS. Twenty-four replicates of increasing numbers of responder T cells (four to six dilution steps) prepared from the spinal cord, the lymph node and the spleen, and 5 × 10⁵ irradiated normal rat thymocytes were incubated with 15 µg/ml MBP, 10 µg/ml MBP72–89 (synthesized in the Queensland Institute of Medical Research) or 5 µg/ml Con A (Sigma) in 96-well U-bottom tissue culture trays. MBP- and MBP72–89-stimulated wells were supplemented with 10 U/ml recombinant human IL-2 (TECINTM, kindly provided
by Hoffmann-La Roche Inc.) 24 h after the commencement of the assay, and incubated for 7 days at 5% CO₂/95% air at 37 °C. Con A-stimulated cultures were not supplemented with IL-2 and were incubated for 5 days. [³H]Thymidine (0.5 µCi) was added to the wells for the last 12 h of culture. The cells were harvested and assayed for incorporated radioactivity. Stimulated wells were scored as positive if the thymidine incorporation was more than twice the mean background level (dilutions of T cells incubated with thymocytes in the absence of antigens or Con A), i.e. when the stimulation index was > 2.0. The frequencies of specifically responding cells were determined as previously described [11]. The limit of sensitivity of the assay was considered to be 1/150 000.

4.7 Statistical analysis

Statistical analysis was performed using one-factor analysis of variance (ANOVA), and either a homoscedastic t-test or a heteroscedastic t-test depending on the result of the F-test.

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